



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>C12N 5/00, C12P 21/00</b> <b>// (C12P 21:00, C12R 11:91)</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 89/ 04867</b> <b>(43) International Publication Date:</b> 1 June 1989 (01.06.89)
<b>(21) International Application Number:</b> PCT/US88/04068 <b>(22) International Filing Date:</b> 14 November 1988 (14.11.88) <b>(31) Priority Application Number:</b> 122,015 <b>(32) Priority Date:</b> 18 November 1987 (18.11.87) <b>(33) Priority Country:</b> US  <b>(71) Applicant:</b> CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). <b>(72) Inventors:</b> MAIORELLA, Brian ; 5661 Broadway, Oakland, CA 94618 (US). INLOW, Duane ; 530 Mariposa Avenue #310, Oakland, CA 94610 (US). HOWARTH, William ; 2524 Brooks Street, El Cerrito, CA 94530 (US).		<b>(74) Agent:</b> HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> METHOD OF INCREASING PRODUCT EXPRESSION THROUGH SOLUTE STRESS  <b>(57) Abstract</b> <p>A method of determining the optimal level of product expression and cell growth of animal cell culture is described. The method generally comprises culturing cells under conditions of solute stress, that is, under conditions whereby optimal cell growth is decreased yet levels of product expression are increased. In a preferred embodiment of the invention is described a method of increasing the yield of monoclonal antibodies comprising culturing hybridoma cells in an environment of solute stress. One approach to the creation of such an environment is the addition of inorganic salts, organic polyols, or metabolic products to the culture medium. One to three fold increases in antibody yield have been obtained by these methods.</p>		

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METHOD OF INCREASING PRODUCT EXPRESSION  
THROUGH SOLUTE STRESS

Field of the Invention

5       The present invention is in the general field of biochemical engineering. More specifically, this invention is in the field of cell and tissue culture dealing primarily with somatic hybrid cell culture.

Background of the Invention

10       With the advent of hybridoma technology and the accompanying availability of monoclonal antibodies, the application of such antibodies has escalated into a variety of areas of the biological sciences. For example, monoclonal antibodies have been used for  
15 the study of cell surface antigens, for affinity purification of proteins, for histocompatibility testing, for studying various viruses and for radioimmunoassay. More recently, it has been recognized that monoclonal antibodies may have medical application for drug targeting and immunotherapy (C.H. Poynton and C.L.  
20 Reading, (1984) Exp Biol 44:13-33). With the increased application of the antibodies in the biological and medicinal sciences, there has come a concomitant demand for high levels of antibody production.  
25

To date, efforts have been undertaken to develop culture conditions to maximize cell culture growth and thereby increase resultant product yield.

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Early work in the development of chemically defined animal cell culture media focused on the formulation of such media to achieve rapid cell proliferation (P.R. White, (1946) Growth 10:231-289, and C. Waymouth (1974) J Natl Cancer Inst 53:1443-1448). Such media incorporate specific nutrients, especially amino acids, vitamins, purines, and pyrimidines. Today some of the more widely used basal media for mammalian cell cultures include Hams F-12, Dulbecco's modified Eagle's medium (DME), RPMI 1640, and Iscove's modified DME. All of these above-referenced basal media are also supplemented with several trace metals and salts, including the major cations (potassium, sodium, calcium, magnesium and the like) with concentration values near isotonic levels. The role of inorganic nutrition in cell culture is discussed in a number of references including R.A. Shooter and G.O. Gey, (1952) Br J Exp Pathol 33:98-103; C. Waymouth, (1974) supra; J.R. Burch and S.J. Pert, (1971) J Cell Sci 8:693-700; R.G. Ham, Growth of Cells in Hormonally Defined Media, Cold Spring Harbor Conferences on Cell Proliferation, Vol. 9, Sato, Pardee and Sirbashin, eds., 1982.

Culture media have been developed specifically for low serum and serum-free mammalian cell cultures for production of monoclonal antibodies. One such serum-free medium is disclosed in European Patent Publication 076,647, published 13 April 1983. Other media have been developed by changing levels of supplements such as trace elements, vitamin and hormone additives wherein variations in the traditional basal media are slight. References to such media include, for example, D. Barnes and G. Sato, (1980) Cell 22:649-655; W.L. Cleveland et al (1983) J Immunol Meth 56:221-234; N. Iscove and F. Melchers, (1978) J Exp Med 147:923-933; T. Kawamoto et al (1983) Anal Biochem 130:445-453; J. Kovar and F. Franek, (1984)

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Immunol Lett 7:339-345; H. Murakami et al (1983) Agric Biol Chem 47(8):1835-1840; H. Murakami et al (1982) Proc Natl Acad Sci USA 79:1158-1162; H. Muzik et al (1982) In Vitro 18:515-524; and S.D. Wolpe, "In Vitro Immunization and Growth of Hybridomas in Serum-Free Medium", in J.P. Mather, ed., "Mammalian Cell Culture," Plenum Press, New York, 1984.

In addition to providing the right kinds and amounts of nutrients, the culture medium must also provide suitable physiochemical conditions. Parameters that are important for clonal growth of hybridoma cell culture include osmolarity, pH buffering, carbon dioxide tension, and partial pressure of oxygen. These all must be adjusted to optimal values for multiplication of each type of cell with, preferably, minimal or no amounts of serum and minimal amounts of protein. Other physical factors such as temperature and illumination must also be controlled carefully.

Efforts to increase antibody yield have focused primarily on means to optimize cell growth and cell density. The optimal conditions for cell growth of mammalian cell culture are generally within narrow ranges for each of the parameters discussed above. For example, typical culture conditions for mammalian hybridoma cell culture use a basal culture medium supplemented with nutritional additives, pH in the range of 6.8 to 7.4 at 35-37°C.

As a general point of reference, antibody titers from murine hybridoma cell lines are highly variable from cell line to cell line and range typically from 10 to 350 ug/ml (K.J. Lambert et al (1987) Dev Indust Microbiol 27:101-106). Human monoclonal antibody expression from human/human or human/mouse fusions are also highly variable from cell line to cell line and range typically from 0.1 to 25 ug/ml

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(R. Hubbard, "Topics in Enzyme and Fermentation Biotechnology," chap. 7, pp. 196-263, A. Wiseman, ed., John Wiley & Sons, New York, 1983). These values are indicative of culture conditions that are optimized for cell growth and cell viability.

Another example from the literature documents that, at least for some cell lines, monoclonal antibody production proceeds even after a culture stops growing (D. Velez et al, (1986) J Imm Methods 86:45-52; S. Reuveny et al, (1986) ibid at p. 53-59). Thus, one strategy for increasing monoclonal antibody yield has been to develop culture conditions that allow growth of hybridomas to higher cell densities and to recover the antibodies late in the stationary phase of cell culture. W. Arathoon and J. Birch, (1986) Science 232:1390-1395 reported that a 1,000 liter hybridoma fermentation produced about 80 grams of monoclonal antibody during the growth phase and another 170 grams of antibody during an extended stationary/death phase. It is not known the means, if any, by which the stationary phase of growth was extended.

Another approach from the literature to increasing antibody production is to achieve high cell densities by cell recycle or entrapment methods. Examples of these methods include hollow fiber reactors (G.L. Altshuler et al (1986) Biotechnol Bioeng XXVIII, 646-658); static maintenance reactors (J. Feder et al, EPA 83870128.2, published 11/7/84); ceramic matrix reactors (A. Marcipar et al (1983) Annals N.Y. Acad Sci 413:416-420); bead immobilized reactors (K. Nilsson et al (1983) Nature 302:629-630); perfusion reactors (J. Feder and W.R. Tolbert, (1985) Amer Biotechnol Lab III:24-36) and others. In some cases, a "resting" cell culture state is reported to be achieved by reducing levels of nutrients in the

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medium (as by reducing serum or protein supplement levels) with antibody production continuing while growth is slowed.

While a variety of methods to increase antibody yield from hybridoma cell culture are being explored, the primary focus is still on the optimization of cell growth. We have discovered that culture conditions for growth optimization and for optimal product expression may differ and that product expression can be increased under conditions of solute stress, created by the addition of certain solutes, notwithstanding the resulting growth inhibitory effects.

The concept of subjecting animal cells, especially mammalian cell cultures, to an environment of solute stress to produce higher product expression yields, such as increased antibody titers, has not been reported. One means for introducing such an environment to the culture is through salt addition which is easily monitored by measuring the osmolarity of the culture medium.

Media osmolarity for mammalian cell culture is usually held in the range of 280-300 mOsm/kg (W.B. Jakoby and I.H. Pastan, Meth Enzymol, vol. LVIII, "Cell Culture", Academic Press (1979), pp. 136-137). Of course, the optimal value may depend upon the specific cell type. For example, as reported in "Tissue Culture, Methods and Applications", edited by P.F. Kruse, Jr., and M.K. Patterson, Jr., Academic Press (1973) p. 704, human lymphocytes survive best at low (about 230 mOsm), and granulocytes at higher osmolarities (about 330 mOsm). Mouse and rabbit eggs develop optimally in vivo at around 270 mOsm, 250-280 mOsm being satisfactory, while above 280 mOsm development is retarded. Iscove reports 280 mOsm to be optimum for growth of murine lymphocytes and hematopoietic

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cells, and Iscoves modified DME is adjusted for this growth promoting osmolarity (N.N. Iscove, (1984) "Method for Serum-Free Culture of Neuronal and Lymphoid Cells," pp. 169-185, Alan R. Liss, ed., New York.

The spread of quality control osmolarity values on a number of commercially available tissue culture media is provided in a table beginning at page 706 in the "Tissue Culture, Methods and Applications" reference, supra. The osmolarity values given therein reflect the 280-300 mOsm/kg range used for mammalian cell culture.

Another means to introduce an environment of solute stress in the cell culture is through the addition of cellular metabolic products, such as lactic acid and ammonia. These products are generally known to be growth inhibitory agents and strategies to reduce the level of these products in the culture medium in order to enhance cell growth have been reported. T. Imamura et al (1982) Anal Biochem 124:353-358; A. Leibovitz, (1963) Am J Hyg 78:173-180; S. Reuveny et al (1986) J Immunol Meth 86:53-59; J.S. Thorpe et al (1987) "The Effect of Waste Products of Cellular Metabolism on Growth and Protein Synthesis in a Mouse Hybridoma Cell Line", Paper #147 presented at American Chemical Society National Meeting, Aug. 30-Sept. 5, 1987, New Orleans, La.--Symposium on Nutrition and Metabolic Regulation in Animal Cell Culture Scale-Up; and M.W. Glacken et al (1986) Biotech & Bioeng XXVIII:1376-1389.

Contrary to the teaching in the art which cautions against major adjustments to culture media osmolarity and other physiochemical parameters, we have found that introducing an environment of solute stress during fermentation can favor an increase in specific (per cell) antibody expression and/or in-



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creased culture longevity which can result in an increase in antibody titer. It is to such a concept that this invention is directed. Briefly, in a preferred embodiment of the invention, an approach to mammalian cell culture which further optimizes yield of antibody production has been developed in which hybridoma cells are cultured under conditions of controlled solute stress. Optionally, the method incorporates prior art advances including the culture of hybrid mammalian cell lines in serum-free media or in high density culture to reduce costs and facilitate purification.

#### Summary of the Invention

Therefore, this invention is directed to a method of determining the optimal level of product expression in animal cell culture wherein the concentration of a solute of interest in a culture medium composition for optimal product expression is different than the culture medium composition determined for optimal cell growth, which method comprises:

- a) growing the animal cell culture in medium to determine optimal cell growth;
- b) varying the concentration of the solute in the culture medium to a concentration above that optimal for cell growth which concentration is effective to create an environment of solute stress on the cell culture;
- c) monitoring the product expression under the varying solute concentrations to determine optimal product expression; and
- d) selecting the solute concentration that provides the optimal combination of cell growth and product expression which allows for optimal productivity.

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In another aspect of this invention is provided a method of increasing the production of monoclonal antibodies during cell culture comprising culturing hybridoma cells under controlled solute stress conditions.

A preferred method of this invention comprises culturing human IgM-producing hybridoma cells.

These and other objects of the invention will be apparent from the following description and claims. Other embodiments of the invention embodying the same or equivalent principles may be used and substitutions may be made as desired by those skilled in the art without departing from the present invention and the purview of the appended claims.

#### Brief Description of the Drawings

Figure 1 shows the effect of 400 mOsm media on antibody yields of D-234 cells in serum-free HL-1 media. The closed circles represent cell growth in 300 mOsm media and the open circles represent the resulting IgM antibody yield. The closed squares represent cell growth in 400 mOsm media and the open squares represent resulting IgM antibody yield.

Figure 2 shows the effect of ammonium chloride on production of antibodies of D-234 cells. The closed circles represent cell growth in the absence of ammonium chloride and the open circles represent the resulting IgM antibody yield. The open triangles represent cell growth in the presence of 10 mM ammonium chloride and the closed triangles represent resulting antibody yield.

#### Description of the Preferred Embodiments

As used herein the term "hybridoma" refers to a hybrid cell line produced by the fusion of a myeloma cell and a plasma cell. The term includes prog-

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eny of heterohybrid myeloma fusions (the result of a fusion with human B cells and a murine myeloma cell line) subsequently fused with a plasma cell, referred to in the art as trioma cell lines.

5 As used herein the term "animal" refers to any mammalian, insect or invertebrate species.

"Mammalian" indicates any mammalian species, and includes rabbits, mice, dogs, cats, primates and humans, preferably humans.

10 As used herein the term "solute" indicates a water soluble agent, including but not limited to inorganic salts and the corresponding ions thereof; organic polyols, including glycerol and sugars such as, for example, glucose, mannose, fructose and  
15 mannitol; and metabolic products such as, for example, lactate or ammonia; which is effective in producing increased product expression.

As used herein the term "solute stress" refers to the addition of solutes in such concentrations, at least above that concentration determined  
20 for optimal cell growth, that produce a growth inhibitory effect or reduced final cell density, that is, a growth rate or maximum cell density less than that determined for optimal growth. However, the  
25 level of product expressed at this reduced growth level is comparatively greater than that level of expression achieved at the optimal growth rate owing to an increase in specific (per cell) product expression rate or an increase in longevity of the culture.

30 As used herein the term "osmolality" refers to the total osmotic activity contributed by ions and nonionized molecules to a media solution. Osmolality, like molality, relates to weight of solvent (mOsm/kg H<sub>2</sub>O) while osmolarity, like molarity, relates to volume  
35 (mOsm/liter solution). Osmolality is one method used to monitor solute stress. Standard osmolality

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refers to the optimum range of clonal growth of mammalian cells which occurs at  $290 \pm 30$  mOsm/kg.

In a preferred embodiment of the invention, methods have been developed for the high level production of mammalian, preferably human, monoclonal antibodies for use as diagnostic reagents or for use in human therapy. In particular, a method of determining the optimal level of product expression in mammalian cell culture has been developed wherein the concentration of a solute of interest in a culture medium composition for optimal product expression is different than the culture medium composition determined for optimal cell growth, which method comprises:

a) growing the mammalian cell culture in medium to determine optimal cell growth;

b) varying the concentration of the solute in the culture medium to a concentration above that optimal for cell growth which concentration is effective to create an environment of solute stress on the cell culture;

c) monitoring the product expression under the varying solute concentrations to determine optimal product expression; and

d) selecting the solute concentration that provides the optimal combination of cell growth and product expression which allows for optimal productivity.

Following the methodology set forth herein, one is able to determine the solute concentration that provides the optimal combination of cell growth and product expression for any particular cell line of interest. Once the solute concentration has been determined, one is able to create an environment of controlled solute stress for culturing the mammalian cell lines and thereby stimulate specific (per cell) product expression and/or increase culture longevity,

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notwithstanding the inhibitory growth effect on the cultured cells.

The mammalian cell culture used in the present invention includes, but is not limited to, any of a number of cell lines of both B-cell and T-cell origin including murine thymic lymphoma cells, human myeloma cell lines, and human lymphoblastoid cells and hybridomas. Accordingly, the product to be optimized includes growth factors, lymphokines, and monoclonal antibodies. The cell cultures may include cell lines which are found to naturally produce such desired products, or have been manipulated by genetic engineering techniques to produce recombinant products.

Solute stress is introduced into the cell culture fermentation by the addition of one or more solutes which effectively inhibit optimal cell growth. The solute can be added at various time periods during the fermentation including prior to, during or after the addition of cells. While such changes to the culture media negatively affect the growth of cultured cells (given the narrow growth parameters known for optimal cell growth) the present invention lies in the discovery that culturing cells in such an environment of solute stress can positively impact specific cell productivity and culture longevity, thereby increasing product yield.

Solute stress which is effective in increasing the product yield can be achieved by increasing the concentration of a solute already present in a culture medium or introducing a new solute to the medium.

In the method of the invention, a sub-lethal solute concentration range is first determined in order to study the solute inhibitory growth effect. This determination is necessary as each cell line may have unique tolerance levels to the selected solute.

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As a second step, various sub-lethal concentrations are studied in more detail to establish the conditions for optimal cell productivity which is responsible for increased product expression. From the data thus generated, one may determine the solute concentration that provides for the optimal combination of cell growth and product expression.

The following discussion, concerning the various types of solutes that may be used in the methods of the present invention, also provides a number of preferred concentration ranges that have been determined for specific hybridoma cell lines. Other cell lines may have somewhat different tolerance levels. These ranges are provided as a guide for determining the optimal combination of growth and product expression levels for a variety of cultured cells and are not to be construed as a limitation of the invention. The concentration ranges provided herein are a good indicator of a possible concentration range for the specific cell line of interest.

The solutes of the invention comprise a number of inorganic salts and ions thereof, including, for example, sodium chloride, potassium chloride, calcium chloride, magnesium chloride and the like, and combinations thereof. Preferred salts include sodium chloride and combinations of sodium chloride and potassium chloride. An effective concentration range for the increased production of monoclonal antibodies by the cell lines D-234 and T-88, using salts such as sodium chloride is 340 to 460 mOsm/kg, with 350 to 400 mOsm/kg being more preferred for the cell line D-234 and 400 to 450 mOsm/kg being more preferred for the cell line T-88.

The concentration values given above, as well as all concentration ranges provided herein regardless of the method of solute concentration meas-

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urement used, have been established prior to the addition of cells. However, the solute may be added before, during or after cell addition. The timing of the solute addition is generally not critical, as it has been found that increasing solute stress by, for example, salt addition, may be performed at various time points during the exponential phase of the growth cycle to achieve an increase in antibody yield. Of course, one skilled in the art will appreciate that the concentration of the metabolic solutes will increase during the course of the fermentation.

In addition to the aforementioned salts, it has been found that solutes which are generally believed to have inhibitory growth effects may also be used in the present invention. For example, lactic acid, a major metabolic end product of glycolysis in hybridoma cell culture, participates in the lowering of the pH during growth, producing sub-optimal growth conditions. The lactate ion itself, may also be growth inhibitory. Efforts have been made to reduce lactic acid production by replacing glucose with alternative sugars (i.e., fructose and galactose) that are less easily metabolized to lactate. It has been assumed that reduction of the level of lactate in the culture medium would enhance both cell growth and antibody production.

However, the present invention demonstrates that the presence of lactate during fermentation can effectively increase antibody yield notwithstanding its inhibitory growth effects. Using the methodology of the present invention, a sub-lethal concentration range (0 to 100 mM sodium lactate) was first determined in order to study the lactate inhibition effect. Various sub-lethal concentrations of sodium lactate are subsequently tested for the effect on product

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expression. For the cell line D-234, an effective concentration range for sodium lactate is 40 to 60 mM.

Ammonia is another substance that has concerned cell culturists due to its negative effects on cell growth. It is produced by cellular metabolism of amino acids as well as by spontaneous decomposition of glutamine. It has been assumed that reduction of ammonia in hybridoma cultures would benefit both cell growth and antibody production. However, as demonstrated herein, an increase in antibody titer was observed despite the inhibition of cell growth in the presence of ammonium chloride. For the cell line D-234, a preferred concentration range for ammonia chloride addition is 3 to 20 mM, with 10-15 mM being more preferred.

The organic polyols useful in the invention include glycerol and a variety of low molecular weight sugars including, for example, glucose, mannose, fructose and mannitol. Of these organic polyols, glucose is preferred, and for the cell line D-234, an effective concentration range for glucose is 6 to 20 g/l, with 7 to 15 g/l being preferred.

The method of the invention is operable with any of a variety of well-known and/or commercially available mammalian cell culture media. Such suitable culture media includes serum-free media such as HL-1 (Ventrex Labs, Portland, ME), HB104 (Hana Biologicals, Berkeley, CA), Iscove's DME medium (Gibco, Grand Island, NY) and RPMI-1640 medium (Gibco) or media supplemented with serum. The hybridomas used in the present method are preferably adapted for growth and maintenance in serum-free medium for large-scale, reproducible spinner culture production of monoclonal antibodies using, for example, a step-wise method.

The method of the invention has been shown to increase antibody titer regardless of the presence



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or absence of serum in the medium. The cell lines used in the present invention may be cell lines of diverse mammalian origin. Rat, mouse and human embodiments are contemplated, with human embodiments illustrated in the examples which follow. The antibodies may be of any class, including IgG and IgM, with IgM types being specifically exemplified herein. The human embodiments are the products of triomas synthesized by somatic cell hybridization using a mouse x human parent hybrid cell line and Epstein-Barr virus (EBV)-transformed human peripheral blood lymphocytes (PBLs) or splenocytes from non-immunized volunteers or volunteers immunized with available Gram-negative bacterial vaccines or inactivated Gram-negative bacteria. Fresh PBLs or splenocytes (not transformed) may be used, if desired.

Briefly, the mouse-human heterohybrid fusion partner designated F3B6 was constructed by fusing human PBL B cells obtained from a blood bank with the murine plasmacytoma cell line NS1 obtained from the American Type Culture Collection (ATCC) under ATCC No. TIB18 (P3/NS1/1-AG4-1). The resulting hybrid cells were adapted for growth in 99% serum-free medium and deposited with the ATCC under ATCC No. HB-8785.

The heterohybrid F3B6 cells and positive EBV-transformed PBL B cells were then used to construct hybridoma cells lines which secrete antibodies illustrative for use in the method of the present invention. A preferred strategy for preparing and identifying such hybrids follows. Cells (PBLs, splenocytes, etc.) are panned on cell-wall lipopolysaccharide (LPS) (an endotoxin of a gram-negative bacteria which produces bacteremia) coated tissue culture plates, then EBV transformed and fused to the tumor fusion partner (mouse myeloma x human B cell or rat myeloma). Panning involves incubation of the popula-

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tion of immunocompetent cells on a plastic surface coated with the relevant antigen. Antigen-specific cells adhere.

Following removal of non-adherent cells, a population of cells specifically enriched for the antigen used is obtained. These cells are transformed by EBV and cultured at  $10^3$  cells per microtiter well using an irradiated lymphoblastoid feeder cell layer. Supernatants from the resulting lymphoblastoid cells are screened by ELISA against an E. coli Rc LPS and a Salmonella Re LPS. Cells that are positive for either Rc or Re lipid A LPS are expanded and fused to a 6-thioguanine-resistant mouse x human B cell fusion partner. If the mouse x human B cell fusion partner is used, hybrids are selected in ouabain and azaserine. Supernatants from the Rc or Re positive hybrids are assayed by ELISA against a spectrum of Gram-negative bacteria and purified Gram-negative bacterial LPSs. Cultures exhibiting a wide range of activity are chosen for in vivo LPS neutralizing activity. Many but not all antibodies so produced are of the IgM class and most demonstrate binding to a wide range of purified lipid A's or rough LPS's. The antibodies demonstrate binding to various smooth LPS's and to a range of clinical bacterial isolates by ELISA.

Two of the hybridoma cell lines which produce the Gram-negative bacterial endotoxin blocking antibodies described above were used to illustrate the methods of the present invention. D-234 and T-88 are representative of hybridomas used in the methods of the present invention to produce increased yields of their respective monoclonal antibodies. D-234 was adapted to growth and maintenance in serum-free medium for large-scale production of monoclonal antibodies. The D-234 hybridoma was created from a fusion of the

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heterohybrid fusion partner F3B6 and human B lymphocytes; a hybridoma sample adapted for growth in serum-free media was deposited with the ATCC under accession number HB-8598. The T-88 hybridoma is a fusion product of the same heterohybrid F3B6 and human splenocytes from a lymphoma patient. A sample of this hybridoma (that was not adapted for growth in serum-free media) was deposited with the ATCC under accession number HB-9431. In addition, a subsequent hybridoma passage of D-234 was deposited with the ATCC under accession number HB-9543. These latter two hybridoma cell lines are specifically exemplified in the following examples.

#### Examples

The following examples are illustrative of this invention. They are not intended to be limiting upon the scope thereof.

#### Example 1

##### Culture of D-234

A one ml ampoule of frozen D-234 stock (ATCC HB-9543) was thawed quickly in a 37°C water bath. The contents were aseptically added to 100 ml prewarmed, pregassed, serum-free HL-1 medium (Ventrex Labs, Portland, Me) supplemented with 0.1% Pluronic® polyol F-68 and 8 mM L-glutamine in a 250 ml Erlenmyer flask with a loosely fitted plastic screw cap. The flask was placed in a humidified incubator (36.5°C, 90% relative humidity and 5% CO<sub>2</sub>) and cultured with shaking at 100-120 rpm.

This parent culture was subcultured during mid-exponential phase, about 2-4 days after inoculation, when the cell density was approximately  $5 \times 10^5$  to  $1 \times 10^6$  viable cells per ml. The subcultures were grown in the daughter flasks under the same culture

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conditions as above, starting with the initial inoculum of  $1 \times 10^5$  and  $5 \times 10^4$  viable cells/ml. The cells were counted using a Coulter Counter, and viability was determined by trypan blue exclusion using an hemocytometer. Maximum total cell densities were around 1.7 million with viable cell densities around 1 million.

For standard batch production, the cultures were allowed to grow to completion which occurs about 7 to 10 days from planting by which time cell viability had declined to 30% or less. The cells were harvested by centrifugation (3,000 rpm for 5 min) to separate the cells and purify the antibodies.

The resulting antibody yield was determined by enzyme-linked immunoadsorbent assay (ELISA) using a standard IgM ELISA but modified by using a high salt (i.e., at least 0.5 M NaCl) assay buffer. IgM titers were around 40 ug/ml.

#### Example 2

##### Effect of Salt Addition on IgM Production In D-234

The following treatments were set up in 100 ml working volume shake flasks at standard planting densities in HL-1 with 0.1% Pluronic F-68 and 8 mM glutamine. A 3.75 M salt solution (27:1 molar ratio NaCl:KCl) was used to increase salt concentration beyond that of the standard HL-1 medium.

Approximately  $1 \times 10^5$  viable cells/ml were used to inoculate the aforementioned culture medium, which was used as the control sample. In addition,  $1 \times 10^5$  viable cells/ml were inoculated into a 400 mOsm initial osmolarity medium. A third sample was formed by inoculating the standard osmolarity medium and, after 88 hours of culture, the 3.75 M salt solution was added to a final concentration of 400 mOsm. At this time point, the cell density was determined indi-

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cating that the culture contained  $\sim 1.2 \times 10^6$  vc/ml. The cells in each of the three cultures were cultured for 9 days, during which time the cell viability and cell density levels were monitored. The IgM titers  
5 were determined for each of the three experimental runs. The results of these experimental runs are provided in Figure 1 and in Table 1 below. As indicated therein, a twofold increase in final IgM titers over  
10 the control ( $\sim 90$  mg/L) was correlated with prolonged viability and increased specific IgM production rates in 400 mOsm cultures where growth rate and cell density are reduced.

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TABLE 1  
D-234 Summary Table

	<u>300 mOsm</u> <u>Control</u>	<u>400 mOsm</u> <u>Initial</u>	<u>"Add Salt"</u> <u>(at 88 Hours)</u>
Maximum Total Cell Density ( $10^5$ /ml)	23	12	22
Maximum Viable Cell Density ( $10^5$ /ml)	15	7.5	14
Ave. Expo- nential Growth Rate $\mu$ (1/hr)	0.033 (0-66 hr)	0.028 (0-89 hr)	0.032 (0-89 hr)
Final IgM Concentration (mg/L)	41	88	58
Ave. Exponen- tial IgM Produc- tion Rate (mg/ $10^9$ /hr)	0.24	0.56	0.40

For the 400 mOsm/kg initial culture, exponential growth rate " $\mu$ " and maximum cell density were reduced, which was indicative of solute stress. The duration of the culture was increased in the high osmolarity culture, and the specific IgM productivity rate was twofold to threefold higher than the control. The extra IgM over and above the control was produced after the peak in viable cell density.

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A 1.5-fold increase in final IgM titer to ~58 mg/L was observed in the culture where salt was added at 88 hours. Specific IgM production rates increased from one day after salt addition into the viable cell decline (versus the control, where production rate declined after the viable cell peak), even though there appeared to be little, if any, difference in the growth curve compared to the control.

For the D-234 cell line, salt addition near the peak viable cell density has an IgM production enhancing effect in the decline phase without any extension of the viable cell curve. This suggests that specific IgM production rates can be increased without slowing growth (and limiting ultimate cell densities) early in culture. However, for D-234, final titers are not as high as those achieved in slow growing (limited cell density) cultures planted in high osmolarity medium.

### Example 3

#### Effect of Inoculation Density and Timing of Salt Addition

Using the methods described in the foregoing examples, the effects of initial inoculation density of D-234 on the specific cell productivity and timing of the salt addition were explored.

A control was run at the standard osmolarity of 300 mOsM medium using  $5 \times 10^4$  planted cultures. These cells exhibited good growth, but viable cell densities were lower than that produced for the  $1 \times 10^5$  cultures (and total cell density of 1.6 versus 1.9 million) with an extension of the viable phase from six to seven days. However, final IgM titers were similar. At 370 mOsM,  $5 \times 10^4$  cells/ml inoculated cultures resulted in significant growth slowing and

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lowering of viable cell density and titers, about half compared with  $1 \times 10^5$  planted cultures.

Various solute stress conditions were tested using the  $5 \times 10^4$  inoculation density culture. Titrers  
5 for 300, 340 and 370 cultures were 40, 75, and 35 mg/L, respectively. It was found that adding salt at day one instead of at day zero to the 370 mOsm allowed the  
10  $5 \times 10^4$  culture to reach viable cell densities ( $5 \times 10^5$  cells/ml) and a titer (65 mg/L IgM) approaching the  $1 \times 10^5$ , 370 culture values ( $6 \times 10^5$  cells/ml and 75 mg/L IgM).

From the results of the previous experiment, 340 and 370 mOsm were chosen as osmolarities to test with salt added on day 0, 1, 2, or 3. The results  
15 indicated that adding salt at different times to the 370 mOsm culture resulted in a slight increase (60 to 65 mg/L final IgM) in final titer concentration.

For the 340 mOsm culture, the 'addition' of salt at day 1 and day 2 led to higher titers (~110 mg/L)  
20 L) than did day 3 addition (~90 mg/L) or day 0 (~70 mg/L).

#### Example 4

##### Effect of Salt Addition on T-88 Growth and IgM Production

25 T-88 cells were grown in replicate 100 ml working volume shake flasks of HL-1 media with 0.1% w/v Pluronic® polyol F-68, 8 mM glutamine and 5% added fetal calf serum at 300 mOsm (control); 340 mOsm; 400 mOsm; and 450 mOsm. Like the above examples,  
30 osmolality was increased by the addition of a 3.75 M salt solution with a 27:1 molar ratio NaCl:KCl. The cultures were grown for 7 days, during which time the cell density and cell viability were periodically monitored.



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Complete growth curves were generated for the control and for the 400 mOsm flasks. The 400 mOsm growth curve showed slow growth and reduced cell density, therefore indicating solute stress had occurred.

5 The duration of the culture was extended, during which IgM production over and above the control was obtained. The specific IgM production rate was higher at 400 mOsm over most of the culture period. Table 2

10 shown below, illustrates that a 30% reduction in total cell density and a 20 to 25% increase in final IgM titer for the 400 and 450 mOsm shake flasks was achieved. IgM produced per million cells from day three to day four was about two times higher at 400

15 and 450 mOsm compared with the control and 340 mOsm treatment. Exponential phase doubling time (Td) for the 400 mOsm treated flasks was higher than for the control (27 versus 20 hours).

Table 2  
T-88 + 5% FCS Summary Table

	<u>Control</u> <u>300</u> <u>mOsm</u>	<u>340</u> <u>mOsm</u>	<u>400</u> <u>mOsm</u>	<u>450</u> <u>mOsm</u>
Maximum Total Cell Density (10 <sup>5</sup> /ml)	23	24	17	17
Final IgM Concentration (mg/L)	37	35	43	46
IgM Produced per Million Cells From Day 3 to Day 4 (ug/10 <sup>6</sup> cells/day)	6	7	15	11
Ave. Exponential Growth Rate mu (1/hr)	0.037 (Td 20)		0.034 (Td 27)	

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Example 5Effect of Lactate on D-234 Growth and IgM Production

This example describes the effect of sodium lactate on growth, viability, and IgM production of D-234.

Approximately  $1 \times 10^5$  cells/ml of D-234 were grown in 250 ml shake flasks (agitated at 100 rpm) in HL-1 medium containing 0.1% Pluronic® polyol F-68 and 8 mM glutamine. A 1 M stock solution of sodium lactate (pH 7.4) in HL-1 was added to the medium. A preliminary screen of the effect of a broad range of sodium lactate concentrations (0-100 mM) on D-234 growth and IgM production was run. It was determined that growth was greatly inhibited by levels of added lactate above 40 mM. Cell densities at day four were reduced at all levels of lactate tested with a critical drop between 40 and 60 mM.

The results of this experiment are given in Table 3 below.

Table 3

Effect of Na Lactate on D-234 Growth  
and IgM Production

Initial Lactate mM	Total Cell Density $1 \times 10^5$ /ml (% Viability)		IgM ug/ml	
	Day 2	Day 4	Day 4	Day 7
0	4.7 (95)	21.0 (90)	10	24
20	5.6 (96)	15.0 (92)	20	35
40	5.1 (92)	12.9 (89)	22	54
60	2.2 (93)	4.1 (87)	19	61
80	2.1 (81)	2.6 (65)	15	28
100	2.2 (72)	2.0 (50)	11	14

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The results indicate that the production of IgM by D-234 was increased with increasing concentrations of sodium lactate up to 60 mM where growth was extremely inhibited, and IgM production peaked at 61 ug/ml compared to the control at 24 ug/ml. Even at 80 mM added lactate, the level of IgM produced was similar to that seen for the control, even though the cell density was only 12% of the control. Specific (per cell) productivity was increased up to 14-fold (at 60 mM added lactate).

#### Example 6

##### Effect of $\text{NH}_4\text{Cl}$ on D-234 Growth and IgM Production

The hybridoma D-234 was grown in HL-1 serum-free medium supplemented with 0.1% Pluronic® polyol F-68, 10 mM glutamine and 10 mM  $\text{NH}_4\text{Cl}$ . A control was also run without  $\text{NH}_4\text{Cl}$ . One hundred ml cultures in 250 ml shake flasks were inoculated at an initial density of  $1 \times 10^5$  viable cells/ml (91% viability).

As illustrated in Figure 2, the addition of 10 mM  $\text{NH}_4\text{Cl}$  inhibited the growth, reduced both viability and the maximum total cell density of the culture ( $2.3 \times 10^6$ /ml for the control vs  $1.1 \times 10^6$ /ml when 10 mM  $\text{NH}_4\text{Cl}$  was added). However, this stress condition prolonged the stationary/decline phase and resulted in a 2-fold increase in the production of IgM.

#### Example 7

##### Effect of High Glucose Concentration on Antibody Production

The hybridoma D-234 was grown in HL-1 medium (Ventrex) which already contains 5.5 g/l. A 500 g/l stock solution of glucose was used to increase the glucose level of the HL-1 medium. The total glucose

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levels tested in this example were 5.5 (control), 10.5, 15.5, and 25.5 g/l.

The 10.5 g/l glucose culture grew more slowly than the control and began to die sooner. While the control reached a maximum of  $8.7 \times 10^5$  viable cells/ml, the 10.5 g/l stressed culture reached  $7.1 \times 10^5$  viable cells/ml. However, the death phase of this culture was longer than the control resulting in higher antibody production: 85 verses 67 mg/l.

The 15.5 g/l glucose culture proved to be very stressful for D-234 resulting in a low maximum viable cell density ( $4.3 \times 10^5$  viable cells/ml) and producing IgM at 50 mg/l. The 25.5 g/l glucose condition proved to be lethal.

#### Deposition of Cultures

The hybridomas used in the above examples to illustrate the method of the present invention were deposited in and accepted by the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland, USA, under the terms of the Budapest Treaty. In addition, the mouse x human fusion partner F3B6 adapted to 99% serum-free medium which partner was the source of these hybridomas was similarly deposited with the ATCC. The deposit dates and the accession numbers are given below:

	<u>Culture</u>	<u>Deposit Date</u>	<u>Accession No.</u>
	D-234	10 August 1984	HB-8598
	D-234	17 September 1987	HB-9543
	T-88	19 May 1987	HB-9431
30	F3B6	18 April 1985	HB-8785

The deposits above were made pursuant to a contract between the ATCC and the assignee of this

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patent application, Cetus Corporation. The contract with ATCC provides for permanent availability of the progeny of these cell lines to the public on the issuance of the U.S. patent describing and identifying the deposit or the publications or upon the laying open to the public of any U.S. or foreign patent application, whichever comes first, and for availability of the progeny of these cell lines to one determined by the U.S. Commissioner of Patents and Trademarks to be entitled thereto according to 35 USC 122 and the Commissioner's rules pursuant thereto (including 37 CFR 1.14 with particular reference to 886 OG 638). The assignee of the present application has agreed that if the cell lines on deposit should die or be lost or destroyed when cultivated under suitable conditions, they will be promptly replaced on notification with a viable culture of the same cell line.

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CLAIMS

1. A method of determining the optimal level of product expression in animal cell culture wherein the concentration of a solute of interest in a culture medium composition for optimal product expression is different than the culture medium composition determined for optimal cell growth, which method comprises:

a) growing the animal cell culture in medium to determine optimal cell growth;

b) varying the concentration of the solute in the culture medium to a concentration above that optimal for cell growth which concentration is effective to create an environment of solute stress on the cell culture;

c) monitoring the product expression under the varying solute concentration conditions to determine optimal product expression; and

d) selecting the solute concentration that provides the optimal combination of cell growth and product expression which allows for optimal productivity.

2. The method of claim 1 wherein the concentration of said solute that provides the optimal combination of cell growth and product expression causes a decrease in cell growth rate or maximum cell density.

3. The method of claim 2 wherein said animal cell culture is a mammalian cell culture.

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4. The method of claim 3 wherein said mammalian cell culture is a hybridoma cell culture that expresses monoclonal antibodies.

5. The method of claim 4 wherein the hybridoma cell culture produces IgM monoclonal antibodies.

6. The method of claim 5 wherein said IgM monoclonal antibody is a human monoclonal antibody.

7. The method of claim 3 wherein the hybridoma is selected from the group consisting of D-234 (ATCC HB-8598), D-234 (ATCC HB-9543), and T-88 (ATCC HB-9431).

8. The method of claim 2 wherein said solute is an inorganic salt or ion thereof.

9. The method of claim 8 wherein said inorganic salt or ion thereof includes sodium chloride and potassium chloride.

10. The method of claim 9 wherein the mammalian cell culture is composed of D-234 cells and the osmolality of the medium with the addition of sodium chloride is in the range of 350 to 400 mOsm/kg.

11. The method of claim 9 wherein the mammalian cell culture is composed of T-88 cells and the osmolality of the medium with the addition of sodium chloride is in the range of 400 to 450 mOsm/kg.

12. The method of claim 2 wherein said solute is a metabolite selected from the group consisting of lactate and ammonium.

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14. A method of increasing the production of monoclonal antibodies during mammalian cell culture comprising culturing hybridoma cells under conditions of solute stress.

15. The method of claim 14 wherein solute stress is produced by an increased concentration of a solute selected from the group consisting of inorganic salts or ions thereof, metabolites, and organic polyols.

16. The method of claim 15 wherein the monoclonal antibodies are human IgM monoclonal antibodies.

17. The method of claim 16 wherein said mammalian hybridoma cell culture is selected from the group consisting of D-234 (ATCC HB-8598), D-234 (ATCC HB-9543), and T-88 (ATCC HB-9431).



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Effect of 400 mOsM  
on IgM Production of D-234

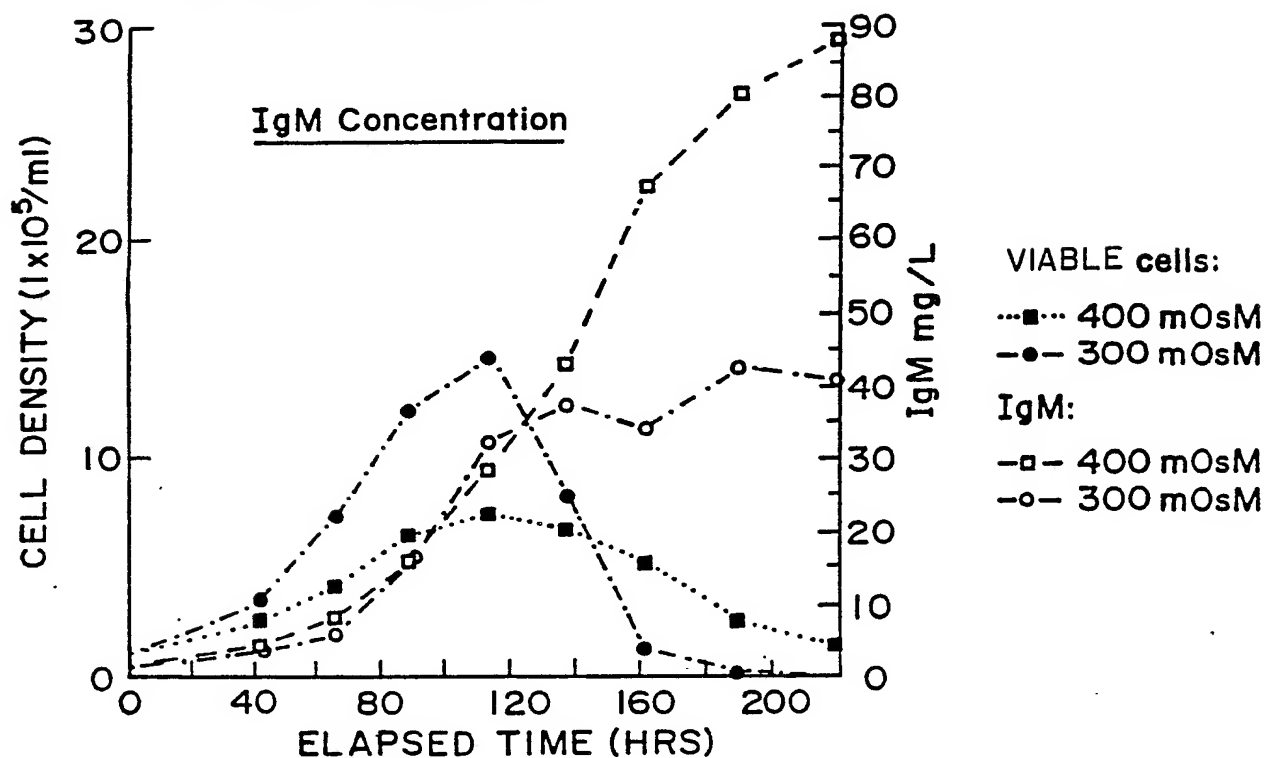
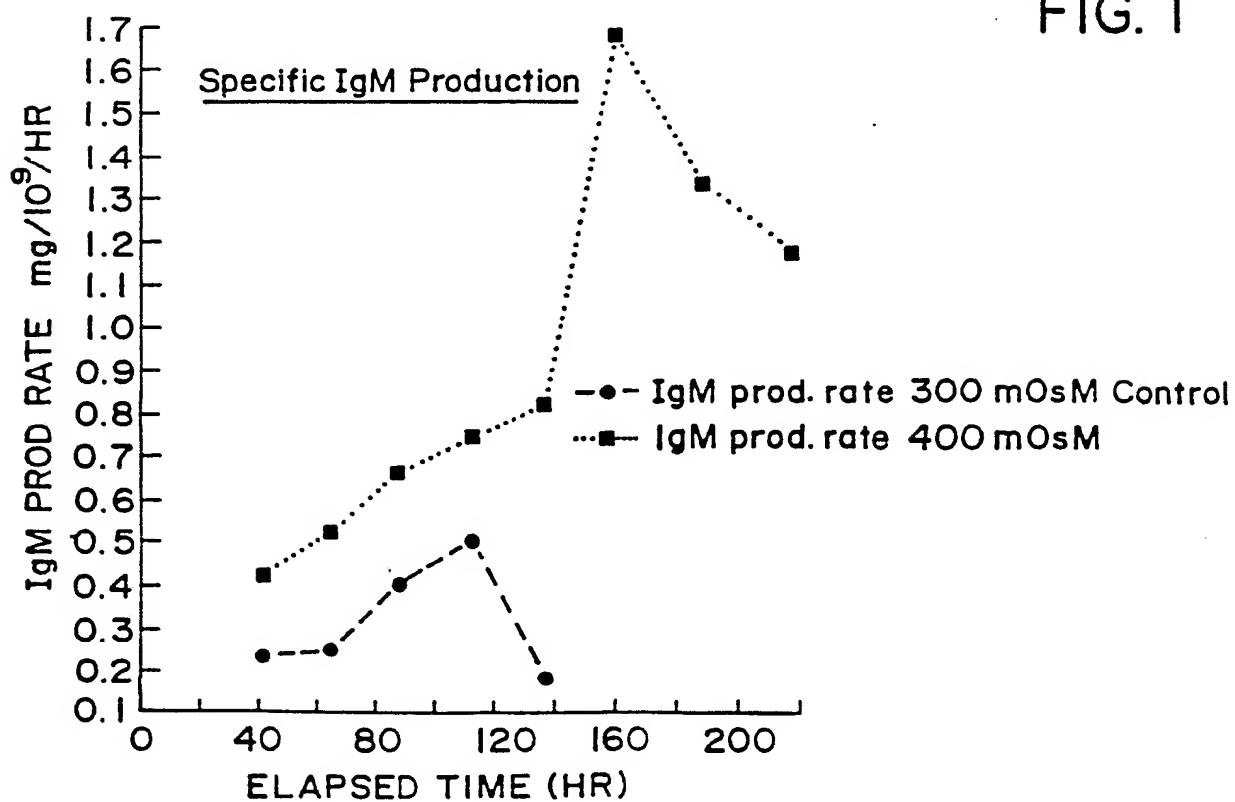


FIG. I



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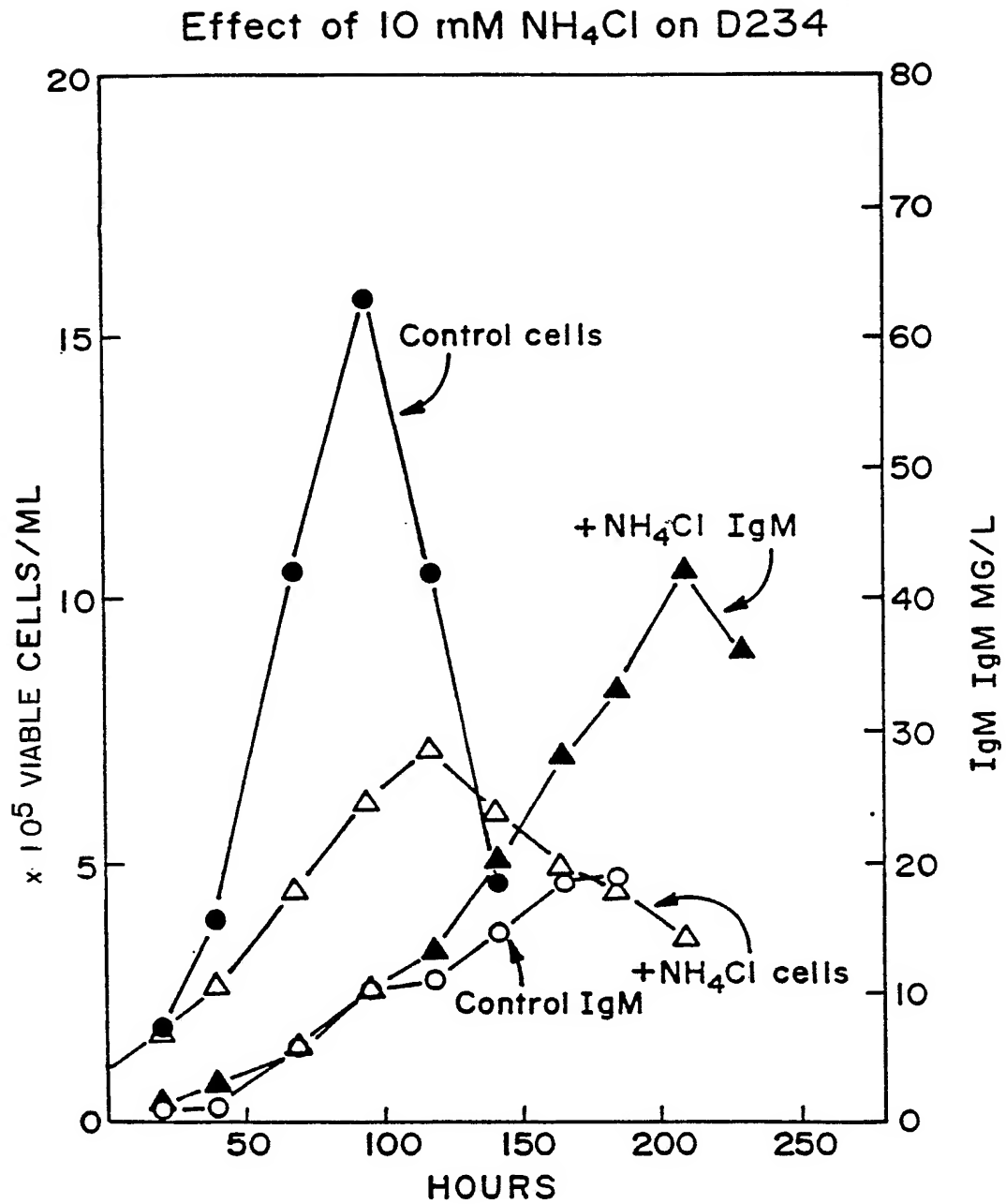
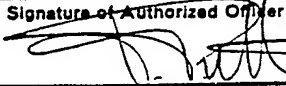
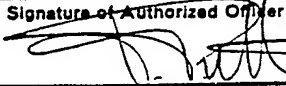
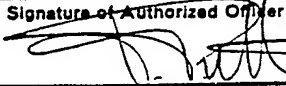


FIG. 2

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 88/04068

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Patent Classification (IPC) or to both National Classification and IPC IPC <sup>4</sup> : C 12 N 5/00; C 12 P 21/00// (C12 P 21/00; C 12 R 11:91)																				
<b>II. FIELDS SEARCHED</b> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Minimum Documentation Searched <sup>7</sup></div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border-right: 1px solid black; padding: 5px;">IPC<sup>4</sup></td> <td style="padding: 5px;">C 12 N</td> </tr> </table> <div style="text-align: center; border-top: 1px solid black; border-bottom: 1px solid black; margin: 5px 0;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup></div>			Classification System	Classification Symbols	IPC <sup>4</sup>	C 12 N														
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<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category <sup>9</sup></th> <th style="border-bottom: 1px solid black;">Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup></th> <th style="width: 15%; border-bottom: 1px solid black;">Relevant to Claim No. <sup>13</sup></th> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">GB, A, 2153830 (DAMON BIOTECH INC.) 29 August 1985, see claims 1-6; example 2</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-11, 14-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">12</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">Y</td> <td style="padding: 5px;">WO, A, 87/00195 (CELLTECH LTD) 15 January 1987, see claims 1, 4, 5, 8; page 4, lines 2-6; page 5, lines 3-17; page 7, lines 22-24; page 8, lines 20-24</td> <td style="text-align: center; vertical-align: top; padding: 5px;">12</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Journal of Immunological Methods, vol. 86, 1986 Elsevier, New York (US) S. Reuveny et al.: "Factors affecting cell growth and monoclonal antibody production in stirred reactors", pages 53-59 (cited in the application)</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-17</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">EP, A, 0101273 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 22 February 1984, see claims 1-3; page 10, lines 8-18</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-17</td> </tr> </table>			Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>	X	GB, A, 2153830 (DAMON BIOTECH INC.) 29 August 1985, see claims 1-6; example 2	1-11, 14-17	Y	--	12	Y	WO, A, 87/00195 (CELLTECH LTD) 15 January 1987, see claims 1, 4, 5, 8; page 4, lines 2-6; page 5, lines 3-17; page 7, lines 22-24; page 8, lines 20-24	12	A	Journal of Immunological Methods, vol. 86, 1986 Elsevier, New York (US) S. Reuveny et al.: "Factors affecting cell growth and monoclonal antibody production in stirred reactors", pages 53-59 (cited in the application)	1-17	A	EP, A, 0101273 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 22 February 1984, see claims 1-3; page 10, lines 8-18	1-17
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<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>10</sup> Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div>																				
<b>IV. CERTIFICATION</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of the Actual Completion of the International Search</td> <td style="width: 50%; border-bottom: 1px solid black; padding: 5px;">Date of Mailing of this International Search Report</td> </tr> <tr> <td style="text-align: center; padding: 5px;">2nd March 1989</td> <td style="text-align: center; padding: 5px;">23. 03. 89</td> </tr> <tr> <td style="border-bottom: 1px solid black; padding: 5px;">International Searching Authority</td> <td style="border-bottom: 1px solid black; padding: 5px;">Signature of Authorized Officer</td> </tr> <tr> <td style="text-align: center; padding: 5px;">EUROPEAN PATENT OFFICE</td> <td style="text-align: center; padding: 5px;">   <b>P.C.G. VAN DER PIJPEN</b> </td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	2nd March 1989	23. 03. 89	International Searching Authority	Signature of Authorized Officer	EUROPEAN PATENT OFFICE	 <b>P.C.G. VAN DER PIJPEN</b>										
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**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8804068  
SA 25594

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 16/03/89  
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA-A- 1206433	24-06-86